

Assessing the Role of Blood Group Genotyping in Predicting Hemolysis in Thalassemia Beta Major Patients with Repeated Transfusions: A Protocol for a Cross-Sectional Study

Vitasari Indriani, Teguh Triyono, Budi Mulyono

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Abstract

Background: Hemolytic transfusion reactions are a major complication in patients with thalassemia beta major receiving regular transfusions. These reactions can be influenced by blood group incompatibilities, particularly in settings with limited genotyping practices. In Indonesia, the role of blood group genotyping in predicting hemolysis has not been thoroughly studied. This study aims to analyze the association between blood group genotyping and the incidence of hemolysis in thalassemia patients undergoing repeated transfusions.

Methods: This is a cross-sectional study involving patients with thalassemia beta major under 18 years old who received regular transfusion with intervals of 2-4 weeks and have received more than 20 units of transfusion. Subjects with leukemia, lymphoproliferative diseases, diabetes, solid tumors, and immunosuppression disorders were excluded from the study. Genotyping examination was conducted using PCR ASP while phenotyping was examined using immunoserology. Follow-up gene sequencing was conducted to observe the blood group variants. Hemolysis was assessed using several markers such as haptoglobin, free hemoglobin, LDH, bilirubin, hemoglobinurine, measured by Cobas C113, ELISA and urinalysis.

Results: Clinical and laboratory data is completed. A total of 90 samples were collected, data analyses are undertaken, and the first results reporting findings is expected to be in September 2024.

Conclusions

The results of this study will provide information on blood groups system that can predict hemolysis occurrence in thalassemia beta major patients with repeated transfusion. This data will contribute to the best possible patient care management and blood transfusion therapy, thereby reducing the risk of hemolysis and improving the quality of life for thalassemia patients in Indonesia.

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Original Manuscript

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Keywords

alloimmunization, phenotype, genotype, hemolysis, thalassemia

Introduction

Background

Beta thalassemia is particularly prevalent in the Mediterranean, Middle East, and Southeast Asia. Annually, over 40,000 babies are born with beta thalassemia, with about 25,500 cases being transfusion-dependent thalassemia (TDT). Patients with TDT require lifelong packed red cell (PRC) transfusions to maintain hemoglobin (Hb) levels between 9 and 11.5 g/dL. In Southeast Asia alone, 20,420 newborns are affected each year, with the Mediterranean and Europe seeing 9,914 and 1,019 cases, respectively, and 341 cases occurring in America. According to the World Health Organization (WHO), Indonesia has a beta thalassemia carrier prevalence of 3–10% per year [1], corroborated by the Ministry of Health of the Republic of Indonesia, which reports that 3.8% of the Indonesian population are carriers [2].

Patients with beta thalassemia who require repeated blood transfusions are at risk of transfusion-related complications, including excessive iron accumulation, alloimmunization, infections, and transfusion reactions [3]. We can divide transfusion reactions into two types: acute/rapid transfusion reactions, which can occur from the time of transfusion to 24 hours after transfusion, and delayed transfusion reactions, which can occur more than 24 hours to 1.5 months after transfusion. Delayed hemolytic transfusion reactions (DHTRs) are particularly concerning thalassemia patients due to the chronic nature of their transfusion requirements. These reactions can lead to significant morbidity, including fever, decreased hemoglobin levels, and elevated markers of hemolysis, such as lactate dehydrogenase (LDH), bilirubin, and free hemoglobin [4]. Free hemoglobin can be measured in the decreased serum haptoglobin, and hemoglobinuria due to intravascular hemolysis [5, 6].

Lifelong blood transfusions can lead to the development of anti-RBC antibodies, including alloantibodies and autoantibodies. The emergence of alloantibodies and autoantibodies to RBC antigens will impede the acquisition of compatible crosses, shorten transfused cells' in vivo survival, increase iron deposition in tissues, and delay the provision of safe transfusions [4]. The homogeneity of donor and recipient populations, RBC phenotype matching policies, and age at transfusion initiation can influence the frequency of alloimmunization, according to research and literature [4, 7-9]. The incidence of alloimmunization in thalassemia is reported to range from 4% to 50%, with anti-E, anti-C, and anti-Kell being the most common alloantibodies identified [7, 8]. These rates are influenced by factors such as donor-recipient population homogeneity, RBC phenotype matching policies, and age at the start of transfusion [4, 7-9].

Current national guidelines in Indonesia for pre-transfusion PRC examination primarily focus on ABO and Rhesus D blood group systems. However, additional clinically significant blood group antigens, such as those within the Kell system, are often not routinely matched, which could increase the risk of alloimmunization and subsequent hemolytic reactions [4, 10]. Moreover, many transfusions do not use leukoreduced products, which further increases the risk of transfusion reactions.

This study addresses the limited comprehensive assessment of blood group genotyping in Indonesia. The primary aim is to assess the association between blood group genotyping and hemolysis in thalassemia patients in the Banyumas region of Indonesia, utilizing molecular genotyping data to provide a more refined understanding of transfusion reactions within this specific population.

The development and application of molecular methods for blood group genotyping in thalassemia patients could provide more precise cross-matching and monitoring of transfusion responses. Genotyping offers a detailed understanding of blood group antigen profiles beyond the standard serological methods, potentially reducing the risk of alloimmunization and improving transfusion safety and outcomes. Understanding the distribution of ABO, Rhesus, and Kell genotypes in the Indonesian thalassemia population and their association with hemolysis could inform clinical practice and guide policy changes towards more personalized transfusion strategies [14, 15].

Additionally, genotype-phenotype discordance can occur in blood group antigens where the genetic data does not align with phenotypic expression due to factors like genetic mutations or epigenetic regulation. Understanding such discordance is crucial as it may affect transfusion outcomes and necessitate more sophisticated diagnostic approaches [16]. Moreover, other genetic factors, such as HLA or non-HLA genes, may influence the immune response to transfusions and impact the risk of hemolysis and alloimmunization. These aspects are essential for a comprehensive understanding of transfusion complications and are potential areas for future research [6].

Aim of the study

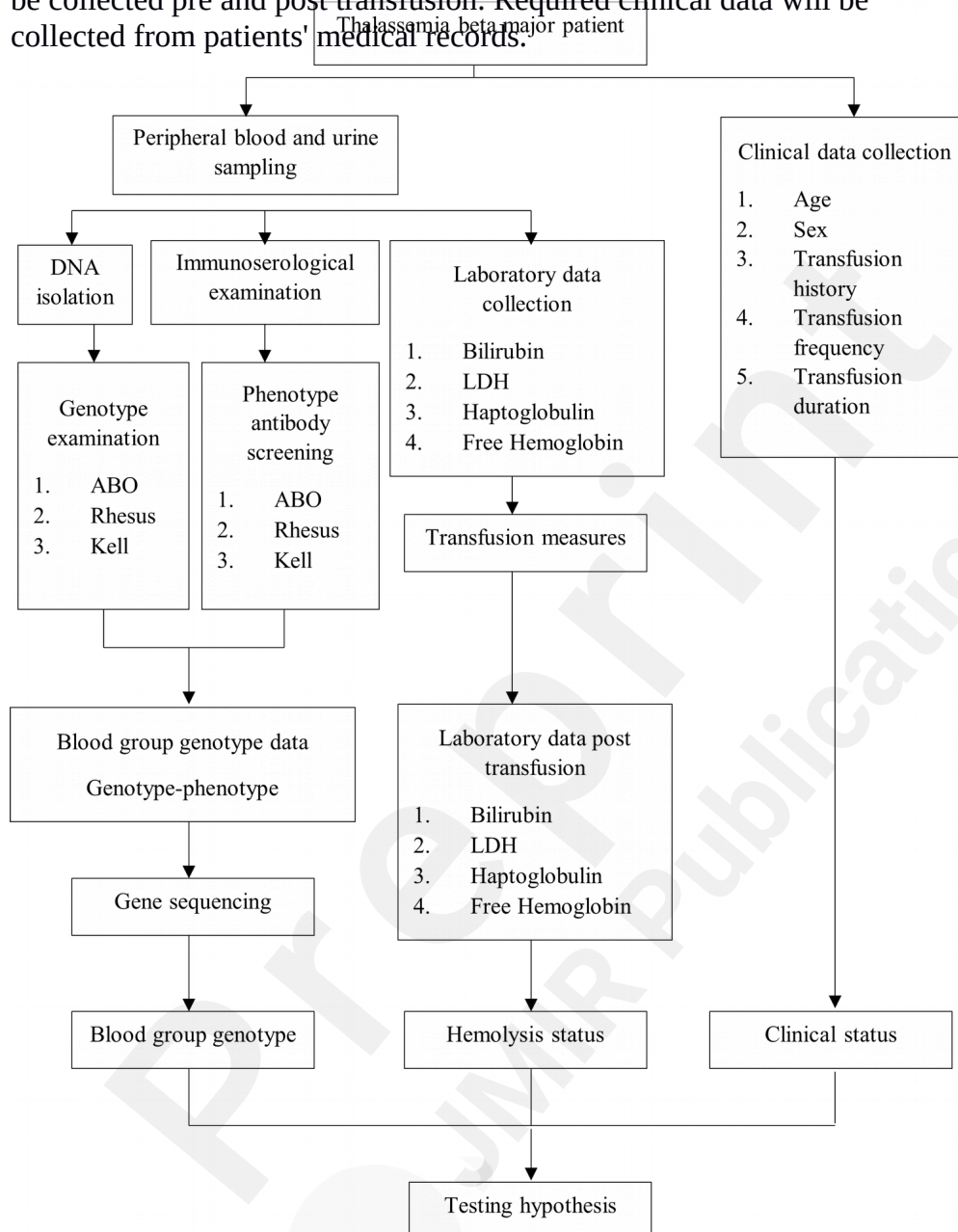
The study aimed to analyze the association of blood group genotyping with the incidence of hemolysis in thalassemia patients undergoing repeated transfusions at Banyumas Regional Hospital, a key referral center for thalassemia care in Indonesia. By identifying specific genotypes that predispose patients to hemolytic reactions, the study seeks to contribute to the development of tailored transfusion strategies that enhance patient care and reduce the risk of adverse outcomes.

Methods

Design

This study is a cross-sectional, observational analytic study designed to assess the association between blood group genotyping and the incidence of hemolysis in thalassemia beta major patients undergoing repeated transfusions. The study follows the STROBE guidelines for reporting observational studies cross-sectional design allows for a snapshot of data to identify potential associations between genotypes and hemolysis at a single point in time, it does not establish causality or capture changes over time [11]. This limitation highlights the need for future longitudinal studies to explore these relationships dynamically and over extended periods. The study procedure is outlined in Figure 1.

Figure 1. Flowchart of participants with thalassemia beta major, eligible participants will be enrolled, and peripheral blood and urine samples will be collected pre and post transfusion. Required clinical data will be collected from patients' medical records.



Setting

The study was conducted at Banyumas Regional Hospital in Central Java, Indonesia, from February 1, 2024, to present. Banyumas Regional Hospital is a referral center for thalassemia care in southern Central Java and surrounding areas, providing an integrated thalassemia service since 2009.

Ethical Considerations

This study adheres to ethical standards for research involving human subjects, as mandated by institutional guidelines and international regulations.

- **Human Subject Ethics Review Approvals:** The study protocol has been approved by the Ethics Committee of the Faculty of Medicine, Public Health, and Nursing at Gadjah Mada University (Approval No. 1511/UN1/FK-KMK.3/S3KK.3/PJ/2023). This approval ensures that all research activities comply with ethical principles for conducting research involving human participants.
- **Informed Consent:** Informed consent was obtained from all participants or their legal guardians prior to participation in the study. Participants were informed of their rights, including the ability to withdraw from the study at any time without any impact on their medical care. For secondary analyses utilizing existing data, we confirm that the original consent covers secondary analysis without requiring additional consent.
- **Privacy and Confidentiality:** All data collected during this study will be anonymized to protect participant identities. Personal identifiers will be removed from datasets, ensuring that individual participants cannot be identified from the data. Data will be stored securely and accessed only by authorized personnel involved in the study.
- **Compensation Details:** Participants will receive a small compensation for their time and participation in the study, ensuring transparency and fairness in the compensation process. The compensation amount will be communicated clearly to participants during the consent process.
- **Identification of Participants in Images:** No identifiable images of participants will be included in this manuscript or supplementary materials. If any images are deemed necessary for inclusion, a statement confirming that consent has been granted from identifiable individuals will be provided, along with relevant consent forms uploaded during resubmission.

Population and Research Subjects

The population of this study were Thalassemia patients who underwent repeated transfusions at Banyumas Hospital. Inclusion and Exclusion criteria are summarized in Textbox 1.

Textbox 1. Inclusion and exclusion criteria for study participation.

Inclusion criteria:

- Age 0 - < 18 years
- Subjects were diagnosed with thalassemia beta major based on history taking, clinical examination and Hb electrophoresis results.
- Received red blood cell transfusions at a minimum interval of 2-4 weeks.
- Subjects had received transfusions of at least 20 units [13].
- Received PRC blood transfusions (store 7 days).

Exclusion criteria:

- Subjects with a diagnosis of leukemia
- Subjects with a diagnosis of lymphoproliferative disease
- Subjects with a diagnosis of diabetes
- Subjects with a diagnosis of solid tumors
- Subjects have immunosuppression disorders.

Calculation of sample size Estimated Proportion (prevalence) in Finite Population / limited population [13]. An assumed prevalence of 8%, based on the study conducted by Kasraian et al. (2022) [14]. The minimum required sample size was determined to be 90 subjects, assuming a confidence level of 95% and a margin of error of 5%. The prevalence estimate was derived from previous studies reporting hemolysis rates in similar patient populations. The final sample size included 134 patients to account for potential dropouts and to ensure adequate power to detect significant associations between blood group genotypes and hemolysis. The sampling method is simple random sampling.

Data Collection and Measurement

Blood Sample Collection: Venous blood samples were taken as much as 6 cc in EDTA and without coagulant tubes, as well as urine samples. Genotyping examination using PCR ASP and phenotyping using immunoserology [15]. Follow-up gene sequencing to see blood group variants [16].

Genotyping and Phenotyping Identification

Genotyping: Blood group genotyping for ABO, Rhesus, and Kell alleles was performed using PCR-ASP. Primers were designed and validated to ensure specificity and efficient amplification. Design criteria included appropriate length, GC content, and melting temperature, with *in silico* verification using NCBI BLAST. Experimental validation involved PCR optimization, amplicon size confirmation by gel electrophoresis, and sequencing for allele confirmation. Quantitative PCR was

used where necessary, with standard curve and melt curve analysis to ensure reliable quantification. [19].

Phenotyping: Immunoserological phenotyping was conducted using the hemagglutination technique. The gel cards used were from Bio-Rad Laboratories, DiaMed GmbH, Switzerland were for the detection of anti-A, anti-B, anti-D, Anti-C, Anti-E, and Anti-K antibodies. Forward grouping was performed by reacting blood cells with antisera, while reverse grouping was done by reacting serum samples with control blood cells. All reagents and techniques were standardized, and external quality controls were implemented to ensure the accuracy of the phenotyping results. Plasma samples that exhibited a positive result with the ID-Diacell antibody screen underwent antibody identification utilizing an extended commercial 11-cell identification panel (ID-DiaPanel) using LISS/Coombs.

Identification of Hemolysis

Markers of hemolysis (haptoglobin, free hemoglobin, LDH, bilirubin, and hemoglobinuria) were examined before and after transfusion [21-23]. Hemolysis was identified using the following cut-off values: plasma free hemoglobin >10 mg/dL, haptoglobin <25 mg/dL, LDH >300 U/L, indirect bilirubin >2.0 mg/dL, and urine hemoglobin >100 ng/mL. These cut-offs were used to assess the severity of hemolysis.

Alloimmunization, defined as the presence of alloantibodies against red blood cell antigens, will be identified using an indirect antiglobulin test (IAT). Positive IAT results will prompt antibody identification (ID-DiaPanel) focusing on clinically significant antigens within the ABO, Rh, and Kell systems.

Data Analysis

The data analysis for this study will involve a comprehensive approach to evaluate the association between blood group genotyping and the incidence of hemolysis in thalassemia beta major patients undergoing repeated transfusions. A descriptive analysis will be conducted for all measured variables, with outliers assessed using boxplots and subsequently excluded from further analyses. The primary measure will be the incidence of hemolysis, evaluated through laboratory markers such as haptoglobin, free hemoglobin, lactate dehydrogenase (LDH), bilirubin, and hemoglobinuria, measured both before transfusion and one-hour post-transfusion. Bivariate analyses using chi-square tests will be employed to analyze the effects of various factors on hemolysis and alloimmunization. Following this, logistic regression models will identify independent predictors of hemolysis while adjusting for potential confounders identified in preliminary analyses. The regression models will include variables found significant in the bivariate analysis, with p-values <0.05 considered statistically significant. Key independent variables include blood group genotypes (ABO, Rhesus, Kell), age, frequency of transfusions, duration of thalassemia diagnosis, underlying medical conditions, and iron chelation therapy status. The selection of these variables was guided by previous literature identifying factors associated with hemolysis and their clinical relevance. Our primary

hypothesis posits that specific blood group genotypes are significantly associated with an increased incidence of hemolysis in thalassemia patients with repeated blood transfusion. Additionally, we hypothesize that clinical factors such as age, transfusion frequency and durations will also significantly influence hemolysis incidence. Missing data will be managed using multiple imputation methods to minimize bias, and sensitivity analyses will compare results from complete-case analyses with those including imputed data to ensure robustness.

Results

Clinical and laboratory data are completed. A total of 90 samples were collected, data analyses are undertaken, and the first results reporting findings are expected to be in March 2025.

Discussion

Principal Findings:

Our study generates baseline data for the Banyumas region in Indonesia, allowing for the development of targeted transfusion strategies tailored to the local population. These strategies contribute to the global understanding of thalassemia management by highlighting the importance of considering regional variations in blood group genetics. Thus, even with the limited sample size, the initial findings have the potential to inform clinical practice and policy changes within Indonesia, laying the groundwork for more personalized transfusion strategies and to global understanding of thalassemia in regions with limited data.

Preliminary findings suggest an association between certain blood group genotypes and higher hemolysis rates in thalassemia patients receiving repeated transfusions. Though complete results are pending, initial data trends support our hypothesis that specific genotypes, especially within the ABO and Rh systems, correlate with increased hemolysis events, likely due to alloimmunization risks tied to blood group antigens [5].

Observed variations in markers such as LDH, bilirubin, and haptoglobin levels in particular genotypes strengthen the case for targeted monitoring. These trends suggest that some blood group genotypes may predispose individuals to hemolytic reactions, reinforcing the importance of considering genotype-matching in transfusion protocols for thalassemia patients. While more data is needed for a conclusive analysis, these early insights indicate that genotype-specific risks should be addressed to enhance transfusion safety and efficacy [14].

Specifically, we anticipate that identifying specific blood group genotypes associated with hemolytic reactions will lead to more personalized transfusion strategies, ultimately improving patient care and outcomes [3]. This approach situates the study's purpose within the broader context of enhancing patient safety and expanding academic knowledge in this critical area of transfusion medicine.

Limitations

This study has identified several limitations. Firstly, the study's sample size of ninety patients may not adequately capture the full genetic and clinical diversity found in larger populations of thalassemia beta major patients in Indonesia or globally. Secondly, the single-center design of this study may introduce selection bias. This could limit the generalizability of the findings. Thirdly, a cross-sectional study offers only a snapshot of data at a single point in time, failing to account for changes over time or establish causality between genotypes and the incidence of hemolysis. *Furthermore, the absence of a cost-effectiveness analysis in this study limits our understanding of the economic implications of implementing genotype-based blood group testing in this population. Future research should address this limitation by conducting a formal cost-effectiveness analysis to evaluate the financial impact of routine genotyping in thalassemia patients.* Therefore, further investigation with larger populations, multi-center studies, and implementing a longitudinal design will be necessary to ensure generalizable findings and help establish causality and track changes over

time.

Future research should investigate the long-term impacts and cost-effectiveness of blood group genotyping, and the genetic basis of alloimmunization in thalassemia. This information can inform policies to integrate genotyping into transfusion protocols, enhancing patient safety and quality of life.

Conclusions

Our study highlights the complex interaction between blood group genotypes and hemolysis risk in thalassemia patients undergoing repeated transfusions. Early findings suggest that certain genotypes, particularly in the ABO, Rhesus, and Kell blood group systems, may be associated with a higher incidence of hemolysis due to alloimmunization. This underlines the importance of exploring molecular genotyping to ensure better transfusion compatibility. Observing variations in hemolysis markers such as LDH, bilirubin, and haptoglobin in relation to specific blood genotypes, our research emphasizes the potential benefits of genotype-matched transfusions. By identifying genotype-specific responses, we aim to reduce hemolysis and improve patient outcomes in long-term transfusion therapy. Further research with a larger cohort will be essential to confirm these preliminary associations and develop targeted transfusion strategies that prioritize genotype compatibility. Ultimately, this study serves as a foundation for implementing genotype-informed transfusion protocols, promising improved care quality and safety for thalassemia patients.

Dissemination Plan

The dissemination plan for this study on the role of blood group genotyping in predicting hemolysis among thalassemia beta major patients undergoing repeated transfusions involves multiple strategies to ensure broad impact and application in clinical practice. We will submit the primary results for publication in high-impact, peer-reviewed medical journals and present them at national and international conferences to engage with experts and foster collaborations. Additionally, workshops and seminars will be conducted at local hospitals and universities to educate healthcare professionals about the study's findings and their practical applications. Policy briefs and recommendations will be formulated for healthcare authorities and policymakers to promote the integration of blood group genotyping into standard transfusion protocols. Collaborating with patient advocacy groups will ensure that information is widely shared through newsletters, social media, and educational materials. Simplified educational materials will be distributed in hospitals, clinics, and support groups. The findings and resources will also be available on open-access platforms and online forums to engage the global thalassemia and transfusion medicine community. This approach aims to inform clinical practice, guide policy development, and enhance patient education, ultimately advancing thalassemia care globally, with a focus on developing countries.

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Authors' Contributions

VI conceived in the study design and drafted this manuscript. TT and BM participated in the study design and critically reviewed the manuscript. All authors read and approved the final manuscript and take full accountability for its content.

Conflicts interests

The author(s) declare that they have no competing interests.

Abbreviations

AABB	: American Association of Blood Banks
ASP	: Allele Specific Primer
AIHA	: Autoimmune Hemolytic Anemia
DAT	: Direct Antiglobulin Test
DHTR	: Delayed Hemolytic Transfusion Reaction
DNA	: deoxyribonucleic acid
ELISA	: Enzyme Linked Immunoabsorbant Essay
EM	: Extended Match
GVHD	: Graft Versus Host Disease
HDN	: Hemolytic Disease of the Newborn
HDFN	: Hemolytic Disease of the Fetus and Newborn
HLA	: Human Leucocyte Antigens
HPLC	: High Performance Liquid Chromatography
HTR	: Hemolytic Transfusion Reaction
Ig G	: Immunoglobulin G
Ig M	: Immunoglobulin M
IL 1	: Interleukin -1
IL 6	: Interleukin- 6
LDH	: Lactate Dehydrogenase
MAC	: Membrane Attack Complex
MALDI-TOF MS	: Matrix assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MCH	: Mean Corpuscular Hemoglobin
MCV	: Mean Corpuscular Volume
NGS	: Next Generation Sequencing
NHFTR	: Nonhemolytic Febrile Transfusion Reactions
NO	: Nitric Oxide
PBM	: Partial Better Match
PCR	: Polymerase Chain Reaction
PRC	: Packed Red Cell
RFLP	: Restriction Fragment Length Polymorphism
SCD	: Sick Cell Disease
RBC	: Red Blood Cell
SNP	: Single Nucleotide Polymorphism
SNV	: Single Nucleotide Variant
SSCP	: Single Strand Confirmation Polymorphism
TDT	: Transfusion Dependent Thalassemia
TIF	: Thalassemia International Federation
TNF	: Tumor Necrosis Factor
TRALI	: Transfusion-related Acute Lung Injury
WE	: Washed Erythrocyte
WHO	: World Health Organization

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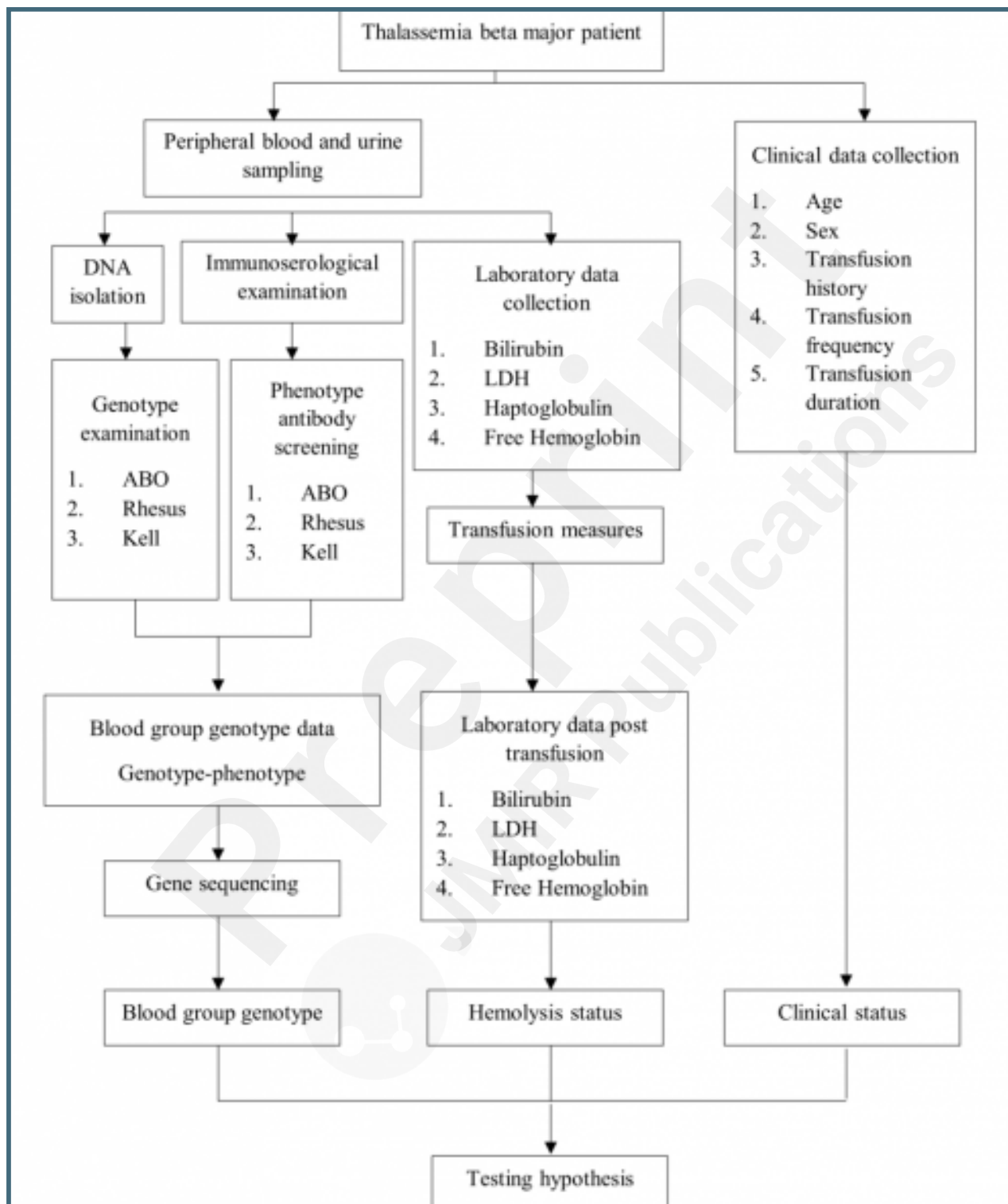
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Supplementary Files

Figures

Flowchart of participants with thalassemia beta major, eligible participants will be enrolled, and peripheral blood and urine samples will be collected pre and post transfusion. Required clinical data will be collected from patients' medical records.



Multimedia Appendixes

Clinical research form.

URL: <http://asset.jmir.pub/assets/5c43d4bcdab33f04d6f73b70dc9a42ca.docx>

STROBE Statement—checklist of items that should be included in reports of observational studies.

URL: <http://asset.jmir.pub/assets/959ebc7388acdb31b5a4f18b2b76d70d.docx>

PCR Primers used in PCR SSP for ABO, Rh and Kell Blood Group Genotyping.

URL: <http://asset.jmir.pub/assets/9d86bba6e525d9f3acf6ff7ee6f87faa.docx>

Protocol For Identification Hemolysis.

URL: <http://asset.jmir.pub/assets/a5bc217aef8a9391b2886cbbcb837f4a.pdf>

Explanation for primer details and cut off value for markers.

URL: <http://asset.jmir.pub/assets/830a517800c95a30f04b7a7a0c3a6846.docx>